ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article *Araştırma Makalesi* DOI: 10.62425/jlasp.1581664

Histopathological and Biochemical Investigation of the Effects of Rutin on Diclofenac-Induced Renal Toxicity in Rats

Sıçanlarda Diklofenak ile Oluşturulan Böbrek Toksisitesinde Rutin'in Etkilerinin Histopatolojik ve Biyokimyasal Olarak Araştırılması

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ABSTRACT

Diclofenac (DCL), which is in the nonsteroidal anti-inflammatory drug (NSAID) category, known for its anti-inflammatory, antipyretic and analgesic properties, has a toxic effect by causing increased oxidative stress and inflammation in tissues when used for a long time. Rutin (RUT) is a flavonoid glycoside with anti-oxidant, anti-inflammatory and anti-apoptotic effects naturally found in many plants. This study aimed to investigate the effects of RUT, a natural antioxidant, on DCL-induced kidney tissue damage. 28 Wistar albino rats were divided into 4 groups: control, DCL, RUT, DCL+RUT100 groups. 100 mg/kg RUT was administered orally for 4 days, and 50 mg/kg DCL was administered intraperitoneally on the 3rd and 4th days. On the 5th day, kidney tissues were taken and oxidative stress, inflammation and apoptotic markers were analyzed by PCR (Polymerase Chain Reaction) method and histopathological analysis of the tissues was performed. Levels of DCL-induced oxidative stress, inflammation and apoptosis parameters in kidney tissues increased compared to the control group ($p<$ .001). With the application of RUT, all these DCL-related increase levels decreased ($p<$ .05). It was concluded that RUT has a potential protective effect against toxicity caused by DCL exposure in kidney tissues.

Keywords: Diclofenac, Nephrotoxicity, Rat, Rutin.

ÖZ

Antiinflamatuar, antipiretik ve analjezik özellikleriyle bilinen nonsteroid antiinflamatuar ilaç (NSAID) kategorisinde yer alan diklofenak (DCL), uzun süreli kullanımı dokularda oksidatif stres ve inflamasyon artışına sebep olarak toksik etki oluşturur. Rutin (RUT), birçok bitkide doğal olarak bulunan anti-oksidan, anti-inflamatuar ve anti-apoptotik etkilere sahip bir flavanoid glikozidtir. Bu çalışmada, doğal bir antioksidan olan RUT'nin DCL kaynaklı böbrek doku hasarı üzerine etkilerinin araştırılması amaçlanmıştır. 28 adet Wistar albino cinsi sıçan kontrol, DCL, RUT, DCL+ RUT100 grupları olmak üzere 4 gruba ayrıldı. 4 gün boyunca 100 mg/kg RUT uygulaması oral yolla verilerek bununla birlikte 3. ve 4. günlerde 50 mg/kg dozda DCL uygulaması intraperitoneal yolla yapıldı. 5. günde böbrek dokuları alındı ve PCR (Polimeraz Zincir Reaksiyonu) yöntemi ile oksidatif stres, inflamasyon ve apoptotik belirteçlerin analizi ve dokuların histopatolojik analizi yapıldı. Böbrek dokularında DCL kaynaklı oksidatif stres, inflamasyon ve apoptoz parametrelerin düzeyleri kontrol grubuna göre artmıştır ($p<$,001). RUT uygulamasıyla birlikte DCL bağlı tüm bu artış düzeylerinde azalmalar meydana gelmiştir ($p<$,05). Böbrek dokularında DCL maruziyetinin sebep olduğu toksisiteye karşı RUT'nin potansiyel koruyucu etkiye sahip olduğu sonucuna varıldı.

Anahtar kelimeler: Diklofenak, Nefrotoksisite, Rutin, Sıçan.

Geliş Tarihi/Received :08.11.2024
Kabul Tarihi/Accepted :25.04.2025
Yayın Tarihi/Publication Date :19.09.2025

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Cite this article: Oğuz Kabayel, R., Akaras, N., Kandemir, Ö., Şimşek, H., & Kandemir, F. M. (2025). Histopathological and Biochemical Investigation of the Effects of Rutin on Diclofenac-Induced Renal Toxicity in Rats. *Journal of Laboratory Animal Science and Practices*, *5*(2), 81-91. https://doi.org/10.62425/jlasp.1581664

Introduction

The kidneys are anatomically and physiologically functional and are quite sensitive to chemical damage compared to other organs due to their high blood flow. The kidneys regulate metabolic functions such as water, electrolyte, and acid-base balance, and also produce hormones, contribute to blood production, and maintain extracellular fluid balance through the renin-angiotensin system while controlling arterial blood pressure (Abiola et al., 2019; Alabi & Akomolafe, 2020).

As a result, the kidneys concentrate toxic chemical substances in the filtrate and transport them along the tubular cells, and some toxic substances are bioactivated (Hickey et al., 2001). In addition, their roles in the metabolism, detoxification, storage, and excretion of drugs and metabolites make the kidneys a target organ vulnerable to damage (Alabi & Akomolafe, 2020; Hickey et al., 2001).

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are among the most commonly prescribed medications with well-known nephrotoxic effects. Diclofenac (DCL), which falls under the NSAID category, is a phenylacetic acid derivative used worldwide by more than 30 million people daily for its anti-inflammatory, antipyretic, and analgesic properties in the treatment of pain, inflammation, degenerative joint disease, rheumatoid arthritis, dysmenorrhea, and trauma (Abiola et al., 2019; Sivaraj and Umarani, 2018). Despite the therapeutic benefits of DCL, it is known to cause nephrotoxicity, cardiotoxicity, hepatotoxicity, gastrointestinal toxicity, and pulmonary toxicity even when used at low doses (Alabi & Akomolafe, 2020).

DCL has a mechanism that leads to the inhibition of cyclooxygenase (COX) enzymes and a decrease in prostaglandin release through the activity of arachidonic acid (Uehara et al., 2016). Recent evidence suggests that the inhibition of COX enzymes may lead to oxidative stress (Thai et al., 2023). The toxicity caused by DCL targets mitochondria by triggering the production of reactive oxygen species (ROS), which leads to apoptosis and DNA damage. While this toxicity can be prevented by the increased expression of antioxidant enzymes against cellular damage caused by ROS, inflammation can be treated by suppressing the activity of the COX enzyme (Prince, 2018). Since the toxic effects of DCL largely arise through oxidative damage mechanisms, scientists have emphasized the importance of natural antioxidants as a solution in the treatment of DCL-induced toxicities (Alabi & Akomolafe, 2020; Prince, 2018). Therefore, it is believed that the use of natural antioxidant compounds will provide significant protection against DCL toxicity.

Flavonoids are a group of natural polyphenolic compounds found in plants; they possess various biological effects and enhance antioxidant enzyme capacity by facilitating the detoxification of free radicals (Alhoshani et al., 2017; Kandemir et al., 2022). Rutin (RUT), which is among flavonoid glycosides, is found in many plants and herbal foods such as oats, buckwheat, tea, pomegranate, apricot, cherry, grapefruit, plum, orange, passionflower, asparagus, grape, fig, and Ruta graveolens, from which it derives its name (Alhoshani et al., 2017; Kandemir et al., 2020). Known as vitamin P, rutoside, quercetin-3-rutinoside, and soforin, this flavonoid has various protective effects under both in vitro and in vivo conditions (Alhoshani et al., 2017; Sirotkin, 2024). In addition to its anti-inflammatory, anti-apoptotic, autophagy-inhibiting, and antioxidant biological effects, it also exhibits pharmacological effects such as nephroprotective, hepatoprotective, anti-allergic, anti-mutagenic, anti-nociceptive, anti-arthritic, anti-cancer, anti-diabetic, anti-ulcer, anti-cholinergic, anti-antibacterial, antifungal, antiviral, and superoxide radical scavenging properties (Çağlayan et al., 2019; Gür & Kandemir, 2023).

In the literature, there is insufficient information regarding the protective effect of RUT against DCL-induced kidney toxicity. Therefore, considering the role of ROS in the toxicity mechanism of DCL, the aim is to investigate the potential defensive effects of RUT, known for its protective properties (antioxidant, anti-apoptotic, and anti-inflammatory), against DCL-induced kidney damage. In this context, oxidative stress, inflammation, and apoptosis markers in kidney tissues will be analyzed using biochemical and molecular methods, and histopathological evaluations will be conducted.

Methods

Experimental Animals

In this study, 28 Wistar albino rats with an average weight of 200-250 grams and aged 10-12 weeks were used, obtained from the KONÜDAM Experimental Medicine Application and Research Center at Konya Necmettin Erbakan University. The animals were kept in a controlled room at a constant temperature of 24-25°C with a 12-hour light-dark cycle (07:00-19:00 light; 19:00-07:00 dark) in cages. The rats were fed with normal drinking water and standard rat chow. They were allowed to rest in their cages for one week to acclimate to the environment before the

experiments began. The ethical approval for this study was granted by the Local Ethics Committee for Animal Experiments of the KONÜDAM Experimental Medicine Application and Research Center at Konya Necmettin Erbakan University, with the decision number 2024-079 dated 25.09.2024.

Experimental Applications

In the study, a total of 28 Wistar albino rats were used, and they were divided into 4 groups with 7 rats in each group. The determination of doses was based on information from the literature (Çağlayan et al., 2019; Varışlı et al., 2023).

1-Control Group: Rats were administered saline solution orally for 4 days, with oral administration on the 3rd and 4th days.

2-RUT Group: RUT was administered orally at a dose of 100 mg/kg for 4 days.

3-DCL Group: DCL was administered intraperitoneally at a dose of 50 mg/kg on the 3rd and 4th days.

4-DCL + RUT100 Group: RUT was administered orally at a dose of 100 mg/kg for 4 days, and DCL was administered intraperitoneally at a dose of 50 mg on the 3rd and 4th days.

Sample Collection

Twenty-four hours after the last application (day 5), the animals were decapitated under light sevoflurane anesthesia, and kidney and blood samples were collected. Blood samples were taken, centrifuged at 3500 rpm for 10 minutes, and stored at -20°C until biochemical analyses. A portion of the kidney tissues was collected for biochemical analyses and stored at -20°C until the analyses were conducted.

Analysis of lipid peroxidation marker

The degree of lipid peroxidation in kidney tissues was assessed by measuring the absorbance of the color generated by the reaction of malondialdehyde (MDA) with thiobarbituric acid at 532 nm. Tissues were homogenized in 1.15% potassium chloride using a homogenizer (Tissue Lyser II, Qiagen). The homogenates were then centrifuged for 15 min at +4°C and 1000g, and the supernatant was used. The technique developed by Kandemir et al. was used to determine MDA levels (Kandemir et al., 2022).

Reverse Transcription PCR (RT-PCR) Analysis

The effects of DCL damage and RUT application on the relative mRNA transcript levels of the gene regions listed in Table 1 were examined using the qRT-PCR technique in kidney tissues collected at the end of the experiment. Total RNA was isolated from the tissues, and cDNA synthesis was performed from the obtained total RNA. The prepared cDNAs, along with primer sequences and MasterMix, were combined to carry out the reaction. The mixture was run in a real-time PCR thermal cycler according to the duration and temperature cycles specified by the manufacturer's instructions. After the completion of the cycles, gene expressions were normalized to β-Actin and evaluated using the 2-ΔΔCT method (Livak & Schmittgen, 2001).

Table 1. Primer Sequences

Tablo 1. Primer Dizileri

Gene	Sequences (5'-3')	Accession no or references PUBMED ID
Cu-Zn SOD	F: AGTCCCGCCCCTTCTAAAAC R: CAATGGCCTCTGTGTAGCCC	PMID: 22057777
CAT	F: ATGGCAACTGTCCCTGAACT R: AGTGACACTGCCTTCCTGAA	PMID: 22057777
GPx	F: CTCGAGTGACAAGCCCGTAG R: ATCTGCTGGTACCACCAGTT	PMID: 22057777
NF-κB	F: AGTCCCGCCCCTTCTAAAAC R: CAATGGCCTCTGTGTAGCCC	NM_001276711.1
TNF-α	F: CTCGAGTGACAAGCCCGTAG R: ATCTGCTGGTACCACCAGTT	NM_012675.3
Caspase -3	F: ACTGGAATGTCAGCTCGCAA R: GCAGTAGTCGCCTCTGAAGA	NM_012922.2
Bax	F: TTTCATCCAGGATCGAGCAG R: AATCATCCTCTGCAGCTCCA	NM_017059.2
Bcl-2	F: GACTTTGCAGAGATGTCCAG R: TCAGGTACTCAGTCATCCAC	NM_016993.2
PERK	F: GATGCCGAGAATCATGGGAA R: AGATTCGAGAAGGGACTCCA	NM_031599.2
ATF-6	F: TCAACTCAGCACGTTCCTGA R: GACCAGTGACAGGCTTCTCT	NM_001107196.1
KIM-1	F:TGGCACTGTGACATCCTCAGA R: GCAACGGACATGCCAACATA	PMID: 32794300
AQP-2	F: AGCTGCCTTCTATGTGGCT R: GCGTTGTTGTGGAGAGCATT	NM_012909.2
β-Actin	F: CAGCCTTCCTTCTTGGGTATG R: AGCTCAGTAACAGTCCGCCT	NM_031144.3

SOD: Superoxide Dismutase, CAT: Catalase, GPx: Glutathione Peroxidase, NF-κB: Nuclear Factor kappa B, TNF-α: Tumor Necrosis Factor-alpha, Caspase-3: Cysteine Aspartate Specific Protease-3, Bax: Bcl-2 Associated X Protein, Bcl-2: B-cell Lymphoma 2, PERK: Protein Kinase R-like Endoplasmic Reticulum Kinase, ATF-6: Activating Transcription Factor 6, KIM-1: Kidney Injury Molecule-1, AQP-2: Aquaporin 2.

Histopathological Analysis

Kidney tissues were collected from anesthetized rats and fixed in a 10% neutral buffered formalin solution for 48 hours. The kidney tissues were washed under running water for one night in accordance with routine tissue processing procedures, and then subjected to dehydration through a series of increasing alcohol concentrations (70% for 1 hour, 80% for 1 hour, 96% for 1 hour, and 99% for 1 hour). The tissues passed through the alcohol series were placed in xylene for a total of one hour in three stages and then treated with paraffin for infiltration. The tissues were subsequently embedded in metal blocks during the blocking stage and formed into solid blocks. Paraffin blocks were sectioned into 4-5 micrometer slices on glass slides using a semi-automatic microtome. The sections on the slides were stained with Hematoxylin and Eosin, a routine tissue stain, for examination. The stained sections were then analyzed using a binocular light microscope and photographed with a camera.

Statistical Analysis

The statistical analysis of the data obtained from the study was conducted using the IBM SPSS software (version 20.0; IBM Corp., North Castle, NY). The Shapiro-Wilk test was used to analyze the sample size, which was less than 50. Since the data had a normal distribution and there were more than two groups, Tukey's multiple comparison test and one-way analysis of variance (ANOVA) were used to compare differences between groups. The results were presented as mean ± SE, with $p < .05$ considered to be statistically significant.

Results

Oxidative Stress Findings

When MDA, one of the markers showing the oxidant status of kidney tissues, was compared with the control group, DCL increased MDA levels ($p < .001$). However, RUT treatment has been determined to reduce MDA levels (Table 2, Figure 1). The mRNA transcript levels of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in kidney tissue were analyzed to assess antioxidant levels (Table 2, Figure 1). In the DCL group, the mRNA transcript levels of SOD, CAT, and GPx decreased compared to the control group ($p < .001$). In the DCL+RUT group, the mRNA transcript levels of SOD, CAT, and GPx increased compared to the DCL group (SOD: $p < .001$, CAT: $p < .01$, GPx: $p < .05$). Accordingly, DCL suppressed the expressions of antioxidant enzymes (SOD, CAT, GPx) in kidney tissue compared to the control group ($p < .001$). These results indicate that DCL may cause tissue damage by reducing the antioxidant capacity in kidney tissues. However, it was observed that RUT treatment increased antioxidant enzyme expressions in the tissue and reduced oxidative damage caused by DCL.

Table 2. MDA Levels and SOD, CAT, GPx mRNA Transcript Levels in Kidney Tissue in All Groups

Tablo 2. Tüm Gruplardaki Böbrek Dokusunda MDA Düzeyleri ve SOD, CAT, GPx mRNA Transkript Düzeyleri

	MDA	SOD	CAT	GPx
Control	17.49±0.48^{a}	1.00±0.02^{c}	1.00±0.01^{c}	1.00±0.06c
RUT	17.18±0.40^{a}	1.13±0.01^{c}	1.12±0.03^{c}	1.07±0.03c
DCL	27.56±0.45^{c}	0.16±0.01^{a}	0.31±0,01^{a}	0.24±0.01^{a}
DCL+RUT	21.19±0.49^{b}	0.60±0.01^{b}	0.55±0.02^{b}	0.79±0.05^{b}

Superscript letters (a, b, c) indicate the difference between groups.$p < .001$

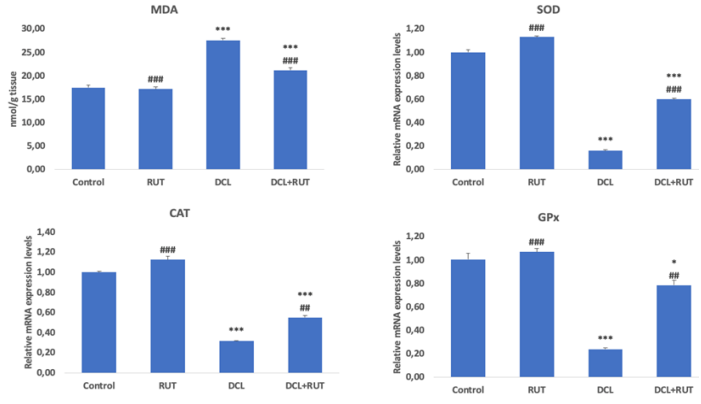

Figure 1: MDA Levels and mRNA Transcript Levels of SOD, CAT, GPx in Kidney Tissues of Rats in Experimental Groups. n=7. ***$p < .001$, *$p < .01$, $p < .05$: Control vs. others; ###$p < .001$, ##$p < .01$, # $p < .05$: DCL vs. others; not significant. (DCL: Diclofenac, RUT: Rutin, MDA: Malondialdehyde, SOD: Superoxide Dismutase, CAT: Catalase, GPx: Glutathione Peroxidase)

Şekil 1: Deney Gruplarındaki Sıçanların Böbrek Dokularındaki MDA Düzeyleri ve SOD, CAT, GPx'in mRNA Transkript Düzeyleri. n=7. ***$p <$,001, *$p <$,01, $p <$,05: Kontrol ve diğerleri; ###$p <$,001, ##$p <$,01, # $p <$,05: DCL ve diğerleri; önemli değil. (DCL: Diklofenak, RUT: Rutin, MDA: Malondialdehit, SOD: Süperoksit Dismutaz, KAT: Katalaz, GPx: Glutatyon Peroksidaz)

Inflammation Findings

To observe the effects of DCL and RUT applications on the inflammatory response in kidney tissues, the mRNA transcript levels of nuclear factor kappa B (NF-κB) and tumor necrosis factor-alpha (TNF-α) were analyzed (Table

3, Figure 2). Accordingly, it was determined that DCL application triggered inflammation by causing an increase in the mRNA transcript levels of NF-κB and TNF-α in the kidneys (p< .001). It was observed that the therapeutic application of DCL+RUT reduced the mRNA transcript levels of NF-κB and TNF-α in kidney tissue (NF-κB: p< .01, TNF-α: p< .001).

Table 3. PERK, ATF-6, NF-κB and TNF-α mRNA Transcript Levels in Kidney Tissue in All Groups.

Tablo 3. Tüm Gruplarda Böbrek Dokusundaki PERK, ATF-6, NF-κB ve TNF-α mRNA Transkript Düzeyleri.

	PERK	ATF-6	NF-kB	TNF-α
Control	1.00±0.09 [a]	1.00±0.39 [a]	1.00±0.01 [a]	1.00±0.07 [a]
RUT	0.93±0.07 [a]	0.85±0.01 [a]	0.90±0.03 [a]	0.86±0.01 [a]
DCL	5.59±0.16 [c]	4.49±0.07 [c]	4.82±0.29 [c]	5.70±0.10 [c]
DCL+RUT	2.99±0.15 [b]	2.36±0.03 [b]	3.27±0.09 [b]	3.22±0.06 [b]

Superscript letters (a, b, c) indicate the difference between groups. p <.001

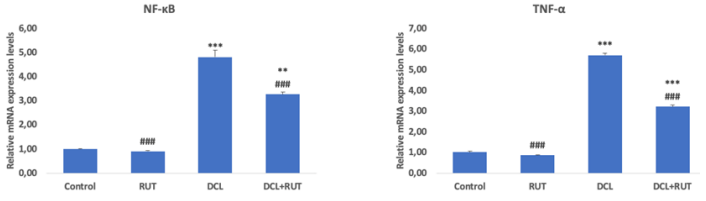

Figure 2: mRNA Transcript Levels of NF-κB and TNF-α in Kidney Tissue of Rats in Experimental Groups. n=7. ***p< .001, *p< .01, p< .05: Control vs. others; ###p< .001, ##p< .01, # p< .05: DCL vs. others; not significant. (DCL: Diclofenac, RUT: Rutin, NF-κB: Nuclear Factor kappa B, TNF-α: Tumor Necrosis Factor-alpha)

Şekil 2: Deney Gruplarındaki Sıçanların Böbrek Dokusunda NF-κB ve TNF-α'nın mRNA Transkript Düzeyleri. n=7. ***p< ,001, *p< ,01, p< ,05: Kontrol ve diğerleri; ###p< ,001, ##p< ,01, # p< ,05: DCL ve diğerleri; önemli değil. (DCL: Diklofenak, RUT: Rutin, NF-κB: Nükleer Faktör kappa B, TNF-α: Tümör Nekroz Faktörü-alfa)

Apoptosis Findings

Table 4 and Figure 3 shows the mRNA transcript levels of cysteine aspartate-specific protease-3 (Caspase-3), Bcl-2-associated X protein (Bax), and B-cell lymphoma 2 (Bcl-2) in rat kidney tissues. According to RT-PCR analysis, DCL was shown to induce Bax and caspase-3 mRNA expressions and to cause apoptosis in the kidney by inhibiting Bcl-2 mRNA expression (p< .001). It was observed that DCL+RUT treatment reduced the mRNA transcript levels of Caspase-3 and Bax in kidney tissue while increasing Bcl-2 mRNA expression (Caspase-3: p< .001, Bax: p< .001, Bcl-2: p< .01). Furthermore, the administration of RUT to the animals provided significant tissue protection by inhibiting the apoptotic pathway in the kidneys.

Table 4. Caspase-3, Bax and Bcl-2 mRNA Transcript Levels in Kidney Tissue in All Groups.

Tablo 4. Tüm Gruplarda Böbrek Dokusundaki Kaspaz-3, Bax ve Bcl-2 mRNA Transkript Düzeyleri.

	Caspase-3	Bax	Bcl-2
Control	1.00±0.16 [a]	1.00±0.01 [a]	1.00±0.01 [c]
RUT	0.91±0.02 [a]	0.93±0.02 [a]	1.09±0.03 [c]
DCL	4.85±0.14 [c]	4.23±0.09 [c]	0.39±0.01 [a]
DCL+RUT	2.79±0.03 [b]	1.99±0.04 [b]	0.66±0.03 [b]

Superscript letters (a, b, c) indicate the difference between groups. p <.001

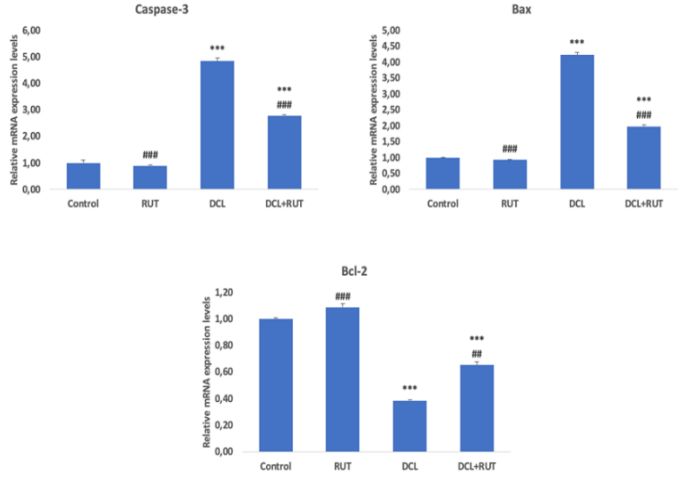

Figure 3: mRNA Transcript Levels of Caspase-3, Bax, and Bcl-2 in Kidney Tissue of Rats in Experimental Groups. n=7. ***p< .001, *p< .01, p< .05: Control vs. others; ###p< .001, ##p< .01, #p< .05: DCL vs. others; ns not significant. (DCL: Diclofenac, RUT: Rutin, Caspase-3: Cysteine Aspartate Specific Protease-3, Bax: Bcl-2 Associated X Protein, Bcl-2: B-cell Lymphoma 2)

Şekil 3: Deney Gruplarındaki Sıçanların Böbrek Dokusunda Kaspaz-3, Bax ve Bcl-2'nin mRNA Transkript Düzeyleri. n=7. ***p< ,001, *p< ,01, p< ,05: Kontrol ve diğerleri; ###p< ,001, ##p< ,01, # p< ,05: DCL ve diğerleri; önemli değil. (DCL: Diklofenak, RUT: Rutin, Kaspaz-3: Sistein Aspartat Spesifik Proteaz-3, Bax: Bcl-2 İlişkili X Proteini, Bcl-2: B Hücreli Lenfoma 2)

Kidney Damage Biomarker Findings

Table 5 and Figure 4 shows the mRNA transcript levels of kidney injury molecule-1 (KIM-1) and aquaporin-2 (AQP-2) in rat kidney tissues. According to RT-PCR analysis, the increase in KIM-1 mRNA transcript expression in the DCL group indicated kidney damage and an inflammatory response (p< .001). On the other hand, the application of RUT in conjunction with DCL was observed to reduce the mRNA transcript levels, thereby suppressing the inflammatory response (p< .001). It was determined that the mRNA expression of AQP-2 significantly decreased due to damage in the kidneys of the DCL-treated group (p< .001). DCL improved the mRNA expression and regulation of AQP-2 in the kidneys of rats treated with RUT (p< .01). This finding suggests that RUT may alleviate the decrease in AQP-2 mRNA expression caused by DCL exposure, potentially providing both protective and reparative effects (p< .01).

Table 5. KIM-1 and AQP-2 mRNA Transcript Levels in Kidney Tissue in All Groups.

Tablo 5. Tüm gruplarda böbrek dokusundaki KIM-1 ve AQP-2 mRNA Transkript Düzeyleri.

	KIM-1	AQP-2
Control	1.00±0.05 [a]	1.00±0.07 [c]
RUT	0.84±0.03 [a]	1.23±0.04 [c]
DCL	3.67±0.11 [c]	0.25±0.01 [a]
DCL+RUT	2.01±0,01 [b]	0.45±0.01 [b]

Superscript letters (a, b, c) indicate the difference between groups. p < .001

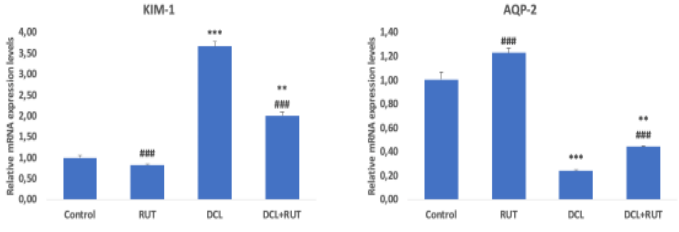

Figure 4: mRNA Transcript Levels of KIM-1 and AQP-2 in Kidney Tissue of Rats in Experimental Groups. n=7. ***p< .001, *p< .01, p< .05: Control vs. others; ###p< .001, ##p< .01, #p< .05: DCL vs. others; ns not significant. (DCL: Diclofenac, RUT: Rutin, KIM-1: Kidney Injury Molecule-1, AQP-2: Aquaporin 2)

Şekil 4: Deney Gruplarındaki Sıçanların Böbrek Dokusunda KIM-1 ve AQP-2'nin mRNA Transkript Düzeyleri. n=7. ***p< ,001, *p< ,01, p< ,05: Kontrol ve diğerleri; ###p< ,001, ##p< ,01, #p< ,05: DCL ve diğerleri; önemli değil. (DCL: Diklofenak, RUT: Rutin, KIM-1: Böbrek Hasarı Molekülü-1, AQP-2: Aquaporin 2)

Endoplasmic Reticulum Stress Findings

The mRNA transcript levels of double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor-6 (ATF-6) genes, which indicate ER stress in rat kidney tissues, are presented in Table 3 and Figure 5. It was observed that DCL caused ER stress by increasing the mRNA transcript levels of ATF-6 and PERK in kidney tissue (p< .001). On the other hand, it was noted that DCL+RUT treatment suppressed the mRNA transcript levels of PERK and ATF-6 in the kidney tissue (PERK: p< .001, ATF-6: p< .01).

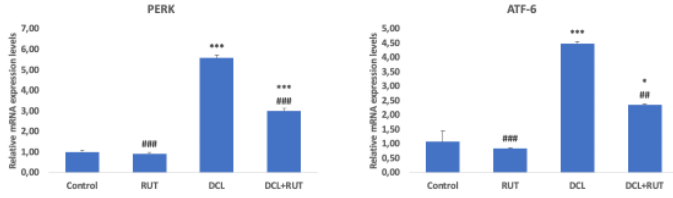

Figure 5: mRNA Transcript Levels of ATF-6 and PERK in Kidney Tissue of Rats in Experimental Groups. n=7. ***p< .001, *p< .01, p< .05: Control vs. Others; ###p< .001, ##p< .01, #p< .05: VPA vs. Others; ns not significant. (DCL: Diclofenac, RUT: Rutin, ATF-6: Activating Transcription Factor-6, PERK: Protein Kinase R-like Endoplasmic Reticulum Kinase)

Şekil 5: Deney Gruplarındaki Sıçanların Böbrek Dokusunda ATF-6 ve PERK'nin mRNA Transkript Düzeyleri. n=7. ***p< ,001, *p< ,01, p< ,05: Kontrol ve Diğerleri; ###p< ,001, ##p< ,01, #p< ,05: VPA ve Diğerleri; önemli değil. (DCL: Diklofenak, RUT: Rutin, ATF-6: Aktive Edici Transkripsiyon Faktörü-6, PERK: Protein Kinaz R-benzeri Endoplazmik Retikulum Kinaz)

Histopathological Findings

The histopathological findings of kidney tissues subjected to H&E staining from four different groups are presented in Figure 6. In the images of the control group rats, the cortex and medulla appeared to have a normal histological structure. The cortex displayed a normal appearance characterized by glomeruli surrounded by convoluted nephron tubules. Additionally, Malpighian corpuscles with normal histological morphology were present, and the structure of the proximal and distal tubules was intact. Only the group that received RUT maintained the normal architecture of the kidney tissue. In the DCL group,

shrunken and atrophic glomeruli in the cortical labyrinth, an enlarged Bowman's space compared to the control, vascular congestion, edema in the interstitium, and inflammatory cell infiltration were notable. Furthermore, DCL induced damage in the proximal and distal renal tubules, leading to the loss of brush borders, epithelial degeneration, and eosinophilic changes in their lumens. In rats treated with RUT, however, improvement in tubular damage was observed compared to the DCL group. In the DCL+RUT group, only mild occlusion in blood vessels and occasional shrunken granules were noted.

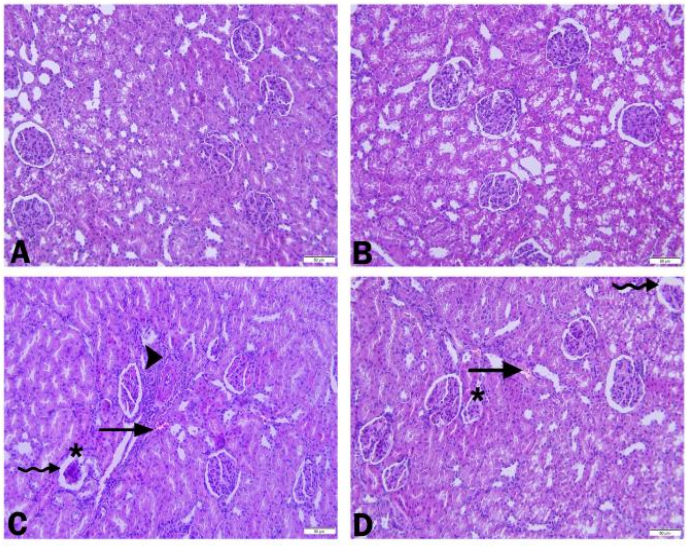

Figure 6: Histopathological Photomicrography of Kidney Sections Stained with Hematoxylin and Eosin (200x). Control (A) and RUT (B) groups show normal histological structure of glomeruli and renal tubules in the cortex. In the DCL (C) group, there is a reduction and degeneration of renal glomeruli (*) with the expansion of the Bowman's space (curved arrow), inflammatory cell infiltration (arrowhead), and vascular congestion (arrow). The DCL + RUT (D) group shows slightly expanded Bowman's space (curved arrow) with reduced and degenerated renal glomeruli (*), and mild vascular congestion (arrow). (DCL: Diclofenac, RUT: Rutin)

Şekil 6: Hematoksilen ve Eozinle boyanmış böbrek kesitlerinin histopatolojik fotomikrografisi (200x) Kontrol (A) ve RUT (B) verilen gruplarının böbrek kesitlerinde, kortekste glomerulus ve renal tübüllerin normal histolojik yapısını gösterir. DCL (C) uygulanan grupta bowman boşluğunun genişlemesiyle (kıvrık ok) büzülmüş ve dejenere olmuş renal glomerülleri (*), inflamatuar hücre infiltrasyonu (ok başı), vasküler konjesyon (ok) gösterir, DCL + RUT (D) grubunda hafif bowman boşluğunun genişlemesiyle (kıvrık ok) büzülmüş ve dejenere olmuş renal glomerüller (*), hafif vasküler konjesyon (ok) gösterir. (DCL: Diklofenak, RUT: Rutin)

Discussion

NSAID DCL can cause toxic effects in many tissues, particularly in the kidneys, at high doses. RUT, on the other hand, is known as a natural flavonoid with strong antioxidant, anti-inflammatory, and anti-apoptotic properties. In this study, the protective effects of RUT against nephrotoxicity induced by DCL were investigated.

An increase in ROS production has been identified as a fundamental cause of various organ dysfunctions in studies conducted (Caglayan et al., 2019; Keles et al., 2014). Furthermore, it has been reported that foods with high antioxidant content in the diet can reduce kidney function losses (Çömez et al., 2024). RUT is known as an antioxidant that has the ability to reduce ROS accumulation and protect cells from damage to prevent oxidative injury. Therefore, in this study, the protective effect of RUT against oxidative stress induced by DCL was investigated by examining the activities of SOD, GPx, and CAT in kidney tissue. It has been reported that organisms prevent cell damage caused by free radicals through mechanisms involving various antioxidant enzymes such as SOD, CAT, and GPx, detoxifying hydrogen peroxide formed in the cells (Akarsu et al., 2023; Çömez et al., 2024). In the study, it was determined that the levels of these three antioxidant enzymes significantly decreased in the kidney tissues of the DCL group compared to the control group. This finding is consistent with the results of a study by Abiola et al., which showed a decrease in SOD, CAT, and GPx enzyme activities in the kidneys of rats treated with DCL, leading to nephrotoxicity by suppressing the antioxidant defense system (Abiola et al., 2019). On the other hand, a study by Kandemir et al. reported that RUT provides significant protection against oxidative stress by increasing the activities of antioxidant enzymes in the kidneys (Kandemir et al., 2022). In our study, the activities of SOD, CAT, and GPx enzymes in the kidney tissues of the DCL+RUT group significantly increased compared to the DCL group. Therefore, it can be said that SOD, CAT, and GPx enzyme activities help prevent oxidative damage caused by DCL in the kidney tissues of rats exposed to DCL. It was concluded that the four hydroxyl groups in the RUT structure probably replenish GSH stores by scavenging free radicals, increase antioxidant enzymes by up-regulating the expression of SOD, CAT, GPx, and reduce MDA levels by alleviating oxidative stress.

Apoptosis, known as programmed cell death, is a pathway directed by various physiological and pathological stimuli and is triggered as oxidative stress levels increase (Akaras et al., 2023; Ayhan et al., 2020; Şimşek et al., 2023). ROS, which play a significant role in apoptosis, are produced in

the mitochondria (Akaras et al., 2023a; Çağlayan et al., 2019). The effect of ROS increases mitochondrial membrane fluidity and permeability (Akaras et al., 2023b). In the apoptosis pathway, these structural and biological changes in the mitochondria are regulated by apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins. While Bcl-2 stabilizes the mitochondrial membrane, Bax increases its permeability (Akaras et al., 2023a; Akaras et al., 2023b). The apoptosis process progresses based on the balance of these proteins. When this balance is disrupted, cytochrome C released from the cytoplasm forms an apoptosome complex with Apaf-1. This complex induces caspase-9, subsequently activating caspase-3, thereby initiating apoptosis (Akaras et al., 2023b; Şimşek et al., 2023). Studies have reported that the disruption of the balance in the Bax/Bcl-2 ratio in kidney tissues leads to increased mitochondrial membrane permeability, resulting in cytochrome C release and caspase-3 activation, thereby reinforcing the notion that this apoptotic effect occurs via the inhibition of the intrinsic pathway mediated by mitochondria (Şimşek et al., 2023). According to our findings, the mRNA transcript levels of the apoptotic genes caspase-3 and Bax increased in the DCL-treated group, while the mRNA transcript level of the anti-apoptotic Bcl-2 gene decreased. These findings suggest that kidney cells inhibit apoptosis via the mitochondrial pathway. In toxicity models created in different organs using various chemicals, RUT has been reported to inhibit apoptotic proteins like caspase-3 and induce anti-apoptotic proteins like Bcl-2, demonstrating an anti-apoptotic effect (Akaras et al., 2023a; Çağlayan et al., 2019). In our study, RUT+DCL group reversed the mRNA levels of these genes. On the other hand, the apoptotic pathway was interrupted, probably due to the alleviating effect of RUT on oxidative stress, ER stress and inflammation. The interruption of apoptosis can be understood from the suppression of Bax and Caspase-3 expressions and the upregulation of Bcl-2 expression.

Inflammation is a biological response of the immune system that can be triggered by various factors such as toxic compounds, damaged cells, and pathogens (Chen et al., 2018). The activation of inflammatory pathways is associated with excessive ROS production (Akaras et al., 2023a). ROS directly activates NF-κB by inducing the phosphorylation following the degradation of the endogenous inhibitor IκB, after which NF-κB is transported from the cytosol to the nucleus, functioning as a transcription factor to stimulate the expression of pro-inflammatory mediators such as TNF-α and inflammatory cytokines like IL-1β (Guo et al., 2024; Kandemir et al., 2022). Previous studies on the reliability of DCL have highlighted that overproduction of ROS can increase the level of NF-κB and induce the expression of pro-inflammatory mediators such as TNF-α resulting (Kankılıç et al., 2024; Wadie et al., 2021). The inhibition of NF-kB is therapeutically important in preventing inflammatory conditions (Bal Taştan et al., 2023). According to the findings of this study, DCL was found to cause an increase in NF-κB and TNF-α mRNA transcript levels, triggering inflammation in kidney tissues, as also confirmed by histological findings. When RUT was administered alongside DCL, a reduction in all these inflammation parameters was observed. Similarly, it has been reported that RUT application prevents inflammation by reducing NF-κB and TNF-α levels (Çağlayan et al., 2019). Kandemir et al. also demonstrated the anti-inflammatory effect of RUT in their in vivo studies with rats (Akaras et al., 2023a; Gür & Kandemir, 2023; Kandemir et al., 2022). Consequently, it is thought that the pathways induced by ROS trigger nephrotoxicity when activated by DCL, while RUT exhibits an anti-inflammatory effect by counteracting this nephrotoxicity through its antioxidant properties.

The kidneys play a vital role in maintaining the body's electrolyte and water balance (Kwon et al., 2013). The ultrafiltrate from the glomerulus (containing substances such as water, sodium chloride, bicarbonates, glucose and amino acids) passes into the proximal tubules, where a significant portion of the kidney's reabsorption function occurs. The reabsorption of water takes place through aquaporin-1 (AQP-1) expressed on the apical and basolateral surfaces of the proximal tubules, and primarily through AQP-2 expressed in collecting duct cells (Musah et al., 2024). Therefore, AQP-2 plays an important role in urine concentration and the body's water balance (Kwon et al., 2013; Musah et al., 2024). Kidney dysfunction and nephrotoxicity significantly affect AQP levels, and it has been reported that AQP-2 levels in the kidney tissues of rats exposed to various toxic agents are significantly reduced (Kwon et al., 2013; Wang et al., 2024). KIM-1, a type I transmembrane glycoprotein, is almost absent in healthy kidney tissue but is highly expressed in damaged proximal and renal tubular cells (Karağaç et al., 2024). Previously, KIM-1 has been reported to be used as a biomarker in both tissue and urine to determine kidney damage caused by various toxic substances (Kim & Moon, 2012). In nephrotoxicity models created in the kidneys using different chemical agents, KIM-1's mRNA and protein levels have been reported to show high expression (Çomaklı et al., 2022; Karağaç et al., 2024). In the current study, it was determined that DCL-induced nephrotoxicity causes kidney dysfunction, indicated by an increase in KIM-1 expression and a decrease in AQP-2 expression. Experimental evidence suggests that RUT restores AQP-2 and KIM-1 levels, indicating its potential use to alleviate the damage caused by DCL.

The endoplasmic reticulum (ER) is involved in protein

biosynthesis, carbohydrate metabolism, drug detoxification and calcium storage in eukaryotic cells (Ricciardi & Gnudi, 2020). The accumulation of misfolded proteins in the ER in response to stimuli such as oxidative stress, metabolic and ischemic damage leads to endoplasmic reticulum stress (Akaras et al., 2024a; Foufelle & Fromenty, 2016; Kandemir et al., 2021).

ER stress triggers the unfolded protein response (UPR), activating PKR-like ER kinase (PERK), transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) to maintain ER homeostasis. When the UPR response is prolonged, cells enter the apoptosis process, leading to significant tissue damage (Akaras et al., 2023c; Akaras et al., 2024b; Kandemir et al., 2021). This response is facilitated by PERK, which reduces the influx of proteins into the ER, alleviating its burden, while ATF6 promotes protein folding and translation, thereby reducing protein accumulation within the ER (Yuan et al., 2024). A study suggested that, alongside important mechanisms such as mitochondrial dysfunction and oxidative stress, endoplasmic reticulum (ER) stress may play a critical role in drug-induced cellular damage, leading to harmful effects such as lipid accumulation, cytolysis, cell death, and inflammation (Foufelle & Fromenty, 2016). In the current study, it was observed that DCL increased the mRNA transcript levels of the ATF-6 and PERK genes, which, consistent with the literature, led to ER stress (Varışlı et al., 2023). Considering the connection between ER stress and oxidative stress, it was hypothesized that RUT could be effective against ER stress; results indicated that RUT could indirectly alleviate ER stress by suppressing oxidative stress. This is supported by the significant reduction in the mRNA transcript levels of the ATF-6 and PERK genes in kidney tissue following RUT treatment. This is thought to be due to the possible reason that DCL-induced ROS causes the accumulation of misfolded proteins due to the acceleration of oxidation of cysteine residues during peroxidation of polyunsaturated fatty acids and formation of disulfide bonds in the ER, thus causing ER stress. Nevertheless, RUT may have reversed this mechanism in the ER by clearing ROS from the kidneys of rats.

The histopathological examination of the kidney plays a critical role in detecting DCL-induced kidney damage. Histopathological evaluations revealed structural abnormalities in kidney tissues from rats, including significantly shrunken and atrophic glomeruli in the cortical labyrinth, expanded Bowman spaces, vascular congestion, prominent edema in the interstitium, and inflammatory cell infiltration. Additionally, degenerative changes such as loss of brush borders in proximal and distal renal tubules, epithelial cell degeneration, and accumulation of eosinophilic material in tubular lumens were observed. The histopathological findings of our study indicate that RUT treatment contributes to the repair of these structural damages caused by DCL.

Conclusion

Our biochemical and histopathological findings strongly confirm that DCL induces toxicity in the kidneys through oxidative stress, inflammation, apoptosis, and endoplasmic reticulum (ER) stress. Additionally, it has been identified that this toxicity leads to a significant decrease in aquaporin-2 (AQP-2) levels. Furthermore, it has been concluded that the antioxidant, anti-inflammatory, and anti-apoptotic effects of RUT are effective against DCL-induced nephrotoxicity, suggesting that RUT holds promise as a potential therapeutic agent for the treatment of kidney toxicity. However, further comprehensive studies are needed to fully understand the underlying molecular mechanisms of RUT's effects.

Ethics Committee Approval: Ethics committee approval was received for this study from the Necmettin Erbakan University, Decision No: 2024-079, Date: 25.09.2024.

Author Contributions: Concept - R.O.K., N.A., F.M.K.; Design - R.O.K., N.A., Ö.K., H.Ş., F.M.K.; Supervision - R.O.K., N.A., F.M.K.; Resources - R.O.K., N.A., Ö.K., H.Ş., F.M.K.; Materials - R.O.K., N.A., Ö.K., H.Ş., F.M.K.; Data Collection and/or Processing - R.O.K., N.A., Ö.K., H.Ş., F.M.K.; Analysis and/or Interpretation - R.O.K., N.A., Ö.K., H.Ş., F.M.K.; Literature Search - R.O.K., N.A., Ö.K., H.Ş., F.M.K.; Writing Manuscript - R.O.K., N.A.;Critical Review - R.O.K., N.A., Ö.K., H.Ş., F.M.K.; Other – R.O.K., N.A., Ö.K., H.Ş., F.M.K.

Peer-review: Externally peer-reviewed.

Funding: Financial support has not been received.

Declaration of Interests: The authors declare that they have no competing interest.

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